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Citation	International journal of food microbiology. 1996. 30(3): 293-302.
Date	1996
URL	http://dx.doi.org/10.1016/0168-1605(96)00955-5; http://hdl.handle.net/1975/533

rRNA gene restriction patterns as a characterization tool for *Lactobacillus sake* strains producing ropy slime

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Abstract

The rRNA gene restriction patterns (ribotypes) of 69 ropy slime producing Lactobacillus sake strains isolated mainly from vacuum-packaged meat products of ten meat plants were determined. Ribotypes of these spoilage bacteria were compared to the corresponding patterns of non-ropy L. sake strains, and also to other species of the genus Lactobacillus, Carnobacterium and Weissella associated with meat products. Ribotyping divided the ropy slime-producing L. sake strains into four characteristic groups corresponding to the phenotypic carbohydrate grouping. The major group was contaminating nine plants located in different parts of Finland and no association between certain ribotypes and individual plants was detected. Differences between ribotypes of slime producing and non-ropy strains of L. sake group sharing the same carbohydrate pattern were not found. Otherwise ribotyping distinguished the ropy slime producing strains from the non-ropy L. sake reference strains. All L. sake strains were distinguished from other species of the genus Lactobacillus, Carnobacterium and Weissella by characteristic banding patterns obtained especially with Hind III digestion. These results suggest that ribotyping is a suitable method for detection and surveillance of the contamination of ropy slime producing L. sake strains but the patterns alone cannot be used as markers of slime production capability. Comparison of ribotypes between different species of the genus Lactobacillus suggest that ribotyping may also be a suitable method for species identification within the genus Lactobacillus.

Key words:Taxonomy; rRNA gene restriction patterns; ribotypes; Ropi-

ness; Meat products, Vacuum-packaged

1. Introduction

Lactic acid bacteria are typically the major microbial group developing on vacuum-packed meat or meat products (Kitchell and Shaw, 1975; Eagan, 1983; Reuter, 1983; Korkeala et al., 1987). The spoilage of meat and meat products caused by lactic acid bacteria is usually manifested by changes in flavour, described as cheesy, sour, acid or liver-like (Pierson et al., 1970; Eagan and Shay, 1982; Eagan, 1983; Korkeala et al., 1987; Schillinger and Lücke, 1987). These changes are believed to be predominantly caused by the accumulation of short-chain fatty acids and of certain ninhydrin-positive compounds as end products formed by lactic acid bacteria (Sutherland et al., 1976; Dainty et al., 1979). Korkeala et al. (1988) were the first to report ropy slime formation in vacuum-packed cooked meat products as a new spoilage problem following lactic acid bacterial contamination. This problem was common in the Finnish meat industry during the years 1987-1990 and it is still occurring sporadically.

Korkeala et al. (1988) and Mäkelä et al. (1992a; 1992b) described five different biochemical profiles for ropy slime producing strains. These profiles were based on carbohydrate fermentation reactions and other phenotypical characteristics. DNA-DNA homology of a representative strain from different phenotypic groups identified four of the groups as Lactobacillus sake strains with the exception of two Leuconostoc amelibiosum strains forming the fifth group (Mäkelä et al., 1992b). L. sake strains form the most important phenotypic bacterial groups causing spoilage by ropy slime.

The present study was undertaken to evaluate the discriminatory power of ribotyping (Grimont and Grimont, 1986) as a charac-

terization tool for the ropy slime producing L. sake strains. These strains have not been characterized by molecular biological typing methods before. As L. sake strains are commonly isolated from vacuum-packaged meat products (Holzapfel and Gerber, 1986; Korkeala and Mäkelä, 1989; Morishita and Shiromizu, 1986) and also used as starter strains, we wanted to obtain characteristic patterns for ropy slime producing L. sake strains. Thus we could distinguish these strains reliably from the other L. sake strains and apply the information from typing results to contamination studies. Ribotyping was selected for the method, because it has been widely used in molecular typing and it has been applied to many different bacterial species, including lactic acid bacteria (Rodrigues et al., 1991). Repeatability of ribotyping is known to be excellent, making long term surveillance and comparison of results between different laboratories possible.

2. Materials and methods

2.1. Bacterial cultures

Ropy slime producing Lactobacillus sake strains were isolated from the raw materials, meat products, air and/or surfaces of ten meat plants from 1987 to 1994. Bacteria were isolated on MRS-S agar (Difco Laboratories, Detroit, USA) and Rogosa SL agar (Orion Diagnostica, Espoo, Finland) as described by Korkeala and Lindroth (1987). The sources of these strains are shown in Table 1. Products spoiled by ropy slime or bacteria isolated from the production environment or materials were sent to our laboratory. One ropy

isolate (Mäkelä and Korkeala, 1992) from each source was accepted for further studies. Since the frequency of sample dispatch varied between the plants, there is no correlation between the rate of contamination and the number of ropy slime producing strains isolated. All of the plants, however, were having current problems with slimy products. These plants were not located in the same area and they were not in physical connection with each other.

The reference strains used in this study are presented in Table 2.

2.2. Detection of ropyness and carbohydrate patterns

Ropy slime producing L. sake strains form ropy colonies when incubated anaerobically on MRS plates (Mäkelä and Korkeala, 1992). This phenomenon together with characteristic carbohydrate patterns (Korkeala et al., 1988; Mäkelä et al. 1992a, 1992b) obtained with the API 50 CH Lactobacillus identification system (Biomerieux, Marcy l'Etoile, France) was used as a selection criterion of ropy slime producing L. sake strain. Cultures were maintained in MRS broth at -70 ^{o}C. When these isolates were subcultured for the API 50 CH Lactobacillus identification and DNA-isolation, seven of the isolates had lost the ability to form ropy colonies. The slime producing ability of each phenotypic group obtained was assured by inoculation tests with vacuum packaged sterile sausages by the method of Korkeala et al. (1988). All the reference L. sake strains were confirmed to be slime-negative by inoculation tests.

2.3. Isolation of chromosomal DNA and restriction enzyme digestion

Cultures were grown overnight in 10 ml MRS broth (Difco, Detroit, USA) at 30 ^{o}C. Cells were harvested from 1 to 1.5 ml depending on cell growth by centrifuging for 2 minutes at full speed (13 000 rpm, about 15 000 x g) in a Biofuge A bench centrifuge (Heraeus Sephatec GmbH, Osterode am Kalkberg, FRG).

Chromosomal DNA was isolated according to the method of Pitcher et al. (1989) with slight modifications. Cells were suspended with TE (Tris-HCl 10 mM, EDTA 1mM) solution containing lysozyme 25 mg/ml and mutanolysin 200 U/ml. The mixture was incubated at 37 ^{o}C for 1 - 2 hrs depending on the resistance of the cell wall.

Preliminary 11 restriction endonucleases (Avr II, Cla I, Eco RI, Hind III, Nar I, Nhe I, Not I, Pvu I, Rsr II, Sac II, Sma I; New England Biolabs, Massachussets, USA) were tested, of which Cla I, Eco RI and Hind III were chosen for clevaging L. sake DNA. 2.5 µg of DNA was cleaved with restriction endonucleases according to the manufacturers instructions. Samples were run overnight (25 V) in 0.8 % agarose gels (SeaKem IDNA agarose, FMC, Rockland, USA) in a GNA 200 apparatus (Pharmacia, Uppsala, Sweden). Digoxigenin labelled phage lambda DNA cleaved with Hind III (Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany) was used as a fragment size marker.

2.4. Preparation of the cDNA probe, Southern transfer and hybridization

A cDNA probe was prepared from E. coli 16S, 23S rRNA (Bohringer Mannheim GmbH) by reverse transcription. The probe was labelled by incorporating digoxigenin-modified dUTP (Boehringer

Mannheim GmbH) by avian myeloblastosis virus reverse transcriptase (Promega, Madison, USA) (Blumberg et al., 1991).

DNA was transferred from gels to MSI Magnagraph membranes (MSI, Westboro, USA) by a VacuGene XL blotting system (Pharmacia) according to the protocol described by the manufacturer. DNA was fixed by UV irradiation in optimal crosslink mode in a Spectrolinker XL 1000 (Spectronics corporation, New York, USA). Membranes were hybridized in a Techne Hybridizer (Techne, Cambridge, United Kingdom) at 68 ^{o}C. Solutions for hybridization, washes and development of the digoxigenin label were performed as described in instructions for DIG DNA Labelling and Detection Kit (Boehringer Mannheim GmbH). Photodocumentation was done by Docugel-V instrument (Scananalytics, Billerica, USA) and RFLPscan software (Scananalytics) was used for fragment size analyses.

2.5. Computer analyses

The ribopatterns of Lactobacillus strains were analyzed by a computed cluster analysis with a BAKT-ID program (Scientific Expert System, Helsinki, Finland). Data from the three banding patterns were combined to produce a set of variables.

3. Results

Table III shows the division of 69 strains producing ropy colonies into four different carbohydrate fermenting groups. Previous phenotypic characterization (Korkeala et al., 1988; Mäkelä et al., 1992a; 1992b) corresponds to these categories. We did not find any new groups. The ability of strains in different groups to pro-

duce ropy slime was confirmed by inoculation tests. All the strains belonging to Groups 2, 3 and 4 were tested and found to produce ropy slime on vacuum packaged sausages. From Group 1 26 randomly selected strains were inoculated and all were found to be slime producing. Seven strains (phenotypically identical to Group 1 strains) which had lost the capability to produce ropy colonies could not produce slime in inoculation tests.

Restriction patterns of the ropy slime producing L. sake strains, L. sake ATCC 15521^{T}, and the two starter strains digested with Eco RI, Hind III and Cla I are presented in Fig. 1. Digestion of the ropy slime producing strains with Eco RI generated 10-13 bands, with Hind III 8-9 bands and with Cla I 7-9 bands from each strain. Ribotyping divided the ropy slime producing L.sake strains into four groups corresponding to the phenotypic grouping. Ribotypes of all the strains having phenotypic Group 1 carbohydrate profile were identical; slime producing strains and seven non-ropy L.sake strains shared the same patterns. The ability to differentiate ropy slime producing L.sake groups varied between the enzymes used. Patterns obtained by digesting with Eco RI and Cla I distinguished the four biochemical groups but Hind III did not separate Group 1 strains from Group 3 strains and Group 2 strains from Group 4 strains. The only difference between strains in Groups 1 and 3 obtained by both the Eco RI and Cla I digests was location of one band in both banding patterns. This difference was slightly easier to interpret from Eco RI patterns (Fig. 1, lanes 1, 4, 15 and 18).

When ropy slime producing strains were compared to non-ropy L. sake reference strains clear differences were noticed. Eco RI or Cla I digests generated again the most distinguishing markers for each strain. Generally the patterns obtained from Eco RI digests

could be used alone when separating the four ropy slime producing groups from non-ropy L. sake strains. The results were clarified, however, using both the Eco RI and Cla I patterns as the basis for the separation. Fig. 2 presents the dendrogram of all the L. sake strains studied based on combined data from all the three enzymes used. Strains were divided into five clusters of which slime producing L. sake strains formed two groups distinguishing abundantly from the L. sake ATCC 15521^T.

rRNA gene restriction fragment analyses clearly distinguished all L. sake strains from the other lactobacilli, Carnobacterium and Weissella strains studied (Fig. 3). In particular, when cleaved with Hind III, the typical banding pattern of two bands sized approximately 1395 and 1305 bp followed by a bandless area, then typically arranged 5-7 bands between 3700 and 2150 bp served as a good marker for all the L. sake strains. Only L. curvatus strains produced bands sized 1395 and 1305 but these strains always had two bands (sized approximately 2140 and 1850 bp) in the area where the L. sake strains did not have any bands (Fig. 4). Eco RI and Cla I digests also produced typical banding patterns for all the L. sake strains. These bands located between the area of 565 and 2030 bp, but there was variation throughout the whole pattern.

4. Discussion

Ribotyping distinguished four groups from L. sake strains causing ropy slime formation in vacuum-packaged meat products. These results agree with previously obtained biochemical typing results

(Korkeala et al., 1988; Mäkelä et al., 1992a; 1992b) and the carbohydrate patterns obtained by API 50 CH Lactobacillus identification system. Ribotyping alone did not distinguish Group 1 ropy slime producing strains from non-ropy strains having the same carbohydrate pattern. The best method for distinguishing L. sake slime producers from non-ropy strains is an inoculation study on a vacuum packaged meat product, whilst other bacteria which cannot produce slime in vacuum-packages can form ropy colonies. However, ropy colony together with characteristic ribotype or carbohydrate pattern seems to be a good marker of slime producing capability.

The four ropy slime producing groups were clearly distinguished from the non-ropy L. sake reference strains. Although ribotyping cannot detect differences among non-ropy and ropy slime producing strains having phenotypic Group 1 carbohydrate pattern, it can be used in monitoring contamination in problematic plants. The type of ropy slime producing L. sake strain can be identified and starter L. sake strains and other non-ropy L. sake strains can be distinguished from the four ropy slime producing groups.

Compared to the other L. sake strains, there was not a clear genetical cluster division between the ropy slime producing L. sake groups and the non-ropy L. sake phenotypes. Comprehensive cluster analysis would, however, require more non-ropy control strains. The ability to produce ropy slime seems not to be restricted genetically into a narrow niche. Exchange of genetic information encoding slime production capability between these strains could explain it, whilst it is not known to be a common feature among L.sake strains to produce ropy slime. We have also noticed that some of the strains originally producing ropy colonies have later lost this characteristic. Curing of plasmids during subculturing may be the

reason for this. It has also been proposed that the ropy phenotypes of Lactococcus lactis subsp. cremoris/lactis are plasmid-encoded functions (von Wright and Tynkkynen, 1987; Neve et al., 1988). Whilst rRNA genes are located in chromosomes only, this method does not distinguish variation in the plasmid profiles. On the otherhand, ribotypes generated by Eco RI and Cla I digestions may not be sensitive enough to detect differences in chromosomal DNA of slime producing and slime-negative strains having Group 1 carbohydrate pattern.

Fig. 3. presents typing results of some of the reference strains used. The ribotypes of Weissella, Carnobacterium and other members of genus Lactobacillus are easy to distinguish from L. sake strains producing ropy slime (Fig. 3, lanes 8 and 16). Typical formations in banding patterns of each species were noticed. Our studies suggest the possibility of distinguishing L. sake strains from other lactobacilli by lower molecular weight fragments of rDNA patterns. In particular the typical banding patterns achieved by Hind III digestion can be used for this purpose. Ribotypes could assist in choosing species specific probes (Chapomier et al., 1987; Hertel et al., 1991; Vogel et al., 1993) for dot blot hybridization tests or strains for DNA-DNA-hybridization assays. The reliable identification of L. sake strains based only on ribotyping needs more studies with confirmed L. sake strains. However, identification of Lactobacillus species by universal probe technique would have technical advantages. Ribotyping has been used previously to identify strains at the level of genetic species within the genuses Aeromonas (Lucchini and Altwegg, 1992), Lactococcus (Rodrigues et al., 1991) and Propionibacterium (Decarvalho et al. 1994). For Lactobacillus spp. restriction endonuclease analyses without probing

the fragments have previously been used as a tool for separating different species (Ståhl et al. 1990).

There is no association between certain ropy slime producing L. sake groups and localized plants or product types. Group 1 strains are most commonly isolated and this group seems to be very homogenic throughout the country. Plasmid profiling and/or more sensitive methods such as pulsed-field gel electrophoresis (Schwartz and Cantor 1984) may detect differences between strains having Group 1 carbohydrate pattern. Although ribotyping can be used as a tool for contamination analyses of ropy slime producing L.sake strains a more revealing characterization method is still needed.

Acknowledgement

We would like to thank Mr Mika Jääskeläinen from Finnzymes Oy for his kind help in photodocumentation and fragment size analyses and Ms Kirsi Ristkari and Mr Pauli Hill for their excellent technical assistance. We are also grateful to Professor Dr G. Reuter and Dr E. Borch for providing us some of the reference strains.

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TABLE I

Number of *L. sake* strains isolated from different sources in meat producing plants

Source	Plant										Total
	A	B	C	D	E	F	G	H	I	J	
Food											
Meat				5							5
Sausage	2	1	9	6	1	1	1	3	1		25
Fermented sausage		2	4					1		7	
Other meat products		1	3	10				3	2	6	25
Production environment											
Air samples				5							5
Surface samples			2	4				2	1		9
Total	2	2	16	34	1	1	1	8	5	6	76

TABLE II

Reference strains

Species	Strain number	Source, if not collection strain
L. sake	ATCC 15521[T]	
	R 105a/67, group A1[a]	Reuter
	BJ 10 strain	Chr. Hansens laboratorium A/S, Horsholm, Denmark
	BITEC LS-25 starter	Gewürzmüller GmbH, Stuttgart, FRG
L. alimentarius	ATCC 29643[T]	
	SMRICC 264	
L. brevis	ATCC 14869[T]	
L. casei subsp. casei	ATCC 393[T]	
L. curvatus	ATCC 25601[T]	
	DSM 20019	
	R 105c/67, group A3[a]	Reuter
	R 102e/67, group A4[a]	Reuter

	R 100/67, group A5[a]	Reuter
	R Rv2eI/67, group A6[a]	Reuter

TABLE II continues

Species	Strain number	Source, if not collection strain
	R IIIj, group A6[a]	Reuter
	Rv40a/67, group A9[a]	Reuter
L. farciminis	DSM 20184^T	
L. plantarum	ATCC 14917^T	
Carnobacterium piscicola	ATCC-35586^T	
Carnobacterium divergens	ATCC-35677^T	
Weissella halotolerans (former Lactobacillus)	ATCC 35410^T	
Weissella viridescens (former Lactobacillus)	ATCC 12706^T	
	SMRICC 193	

[a] bacterial groups A1 - A9 described by Reuter (1970)

ATCC = American Type Culture Collection

DSM = Deutsche Samlung für Mikroorganismen

SMRICC = Swedish Meat Research Culture Collection

TABLE III

Positive reactions of API 50 CH Lactobacillus identification system of ropy slime producing L. sake strains

Group (number of strains)	Acid formed	Detected in plants
1 (60)[a]	Ribose, galactose, D-glucose, D-fructose, D-mannose, N-acetyl glucosamine, esculine, cellobiose, melibiose, sucrose, trehalose, β-gentiobiose	A, B, C, D, E, F, H, I, J
2 (2)	Ribose, galactose, D-glucose, D-fructose, D-mannose, **α-methyl-D-mannoside**[b], N-acetyl glucosamine, esculine, salicine, maltose, melibiose, sucrose, trehalose, **gluconate**	C
3 (6)	Ribose, galactose, D-glucose, D-fructose, D-mannose, N-acetyl glucosamine, esculine, maltose, melibiose, sucrose, trehalose	D, I
4 (1)	L-arabinose, ribose, galactose, D-glucose, D-fructose, D-mannose, N-acetyl glucosamine, **arbutine**, esculine, salicine, cellobiose, melibiose, sucrose	G

[a]Seven strains (from plants C and D) having Group 1 carbohydrate pattern had lost the ability to produce ropy colonies and slime in inoculation tests. These strains are not included in this table.

Fig. 1. rRNA gene restriction patterns. 1-7 Eco RI digests, 8-14 Hind III digests and 15-21 Cla I digests. 1, 8 and 15 Group 1 strain; 2, 9 and 16 Group 2 strain; 3, 10 and 17 Group 4 strain; 4, 11, and 18 Group 3 strain; 5, 12 and 19 L.sake ATCC 15521^{T}; 6, 13 and 20 L. sake from BITEC-LS 25 starter and 7, 14, and 21 L. sake BJ 10 starter strain.

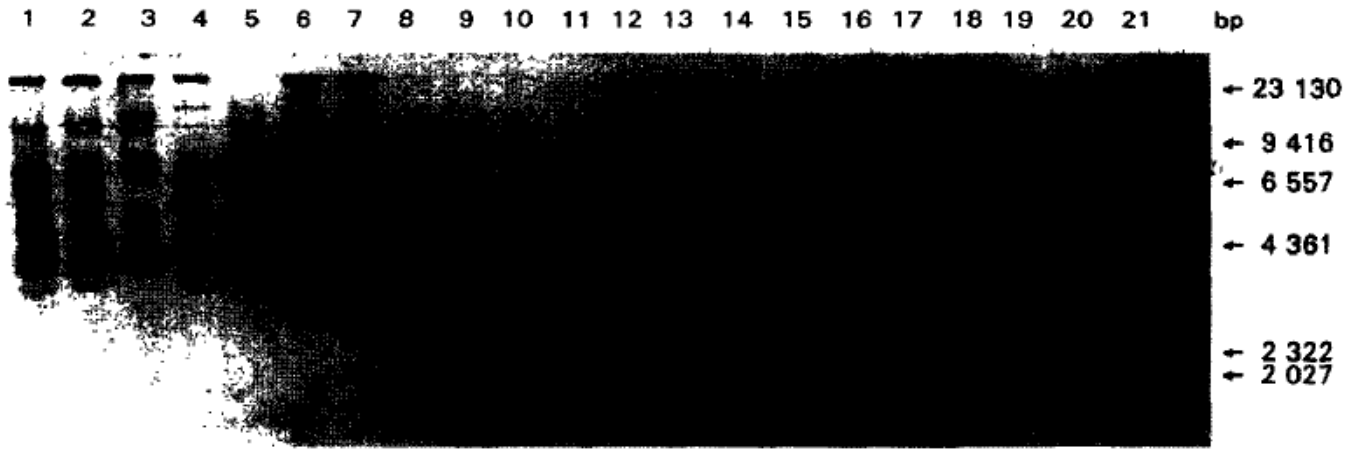

Fig. 2. Dendrogram of L. sake strains created with rRNA gene restriction patterns obtained by cleavaging with Eco RI, Hind III and Cla I. Data from the three banding patterns were combined to produce a set of variables.

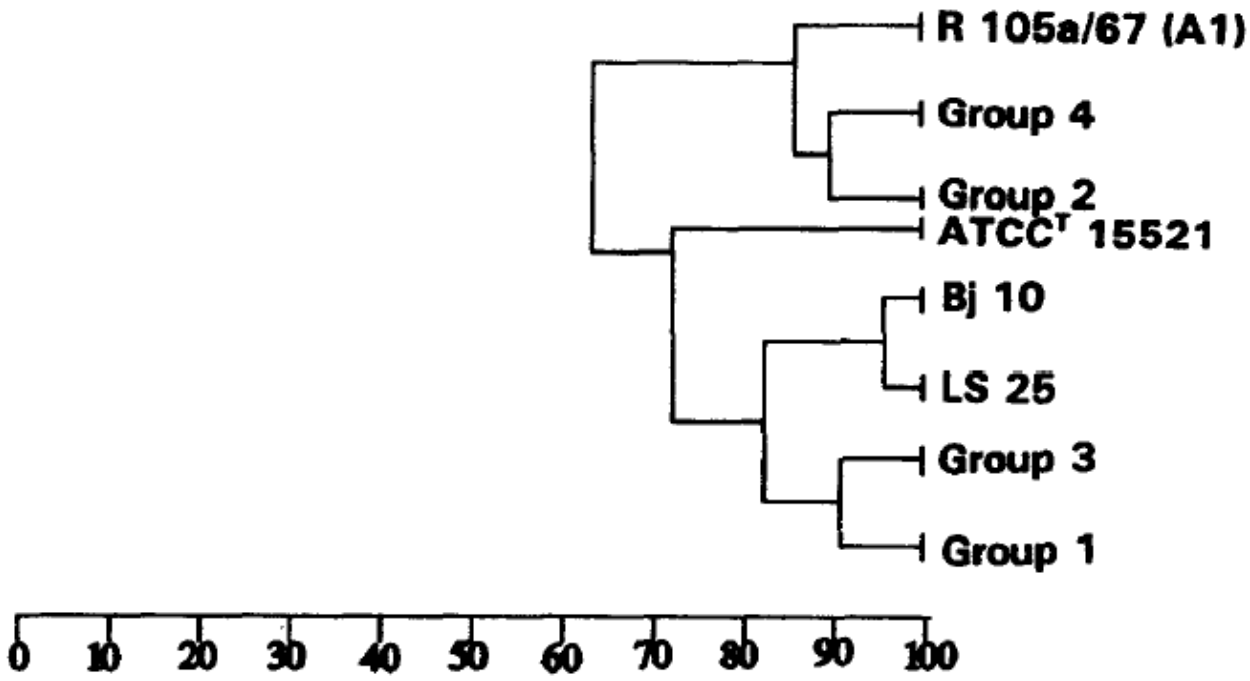

Fig. 3. Typical arrangement of bands obtained with Hind III digestion of L. sake (lanes 1 and 4) and L. curvatus strains (lanes 2 and 3).

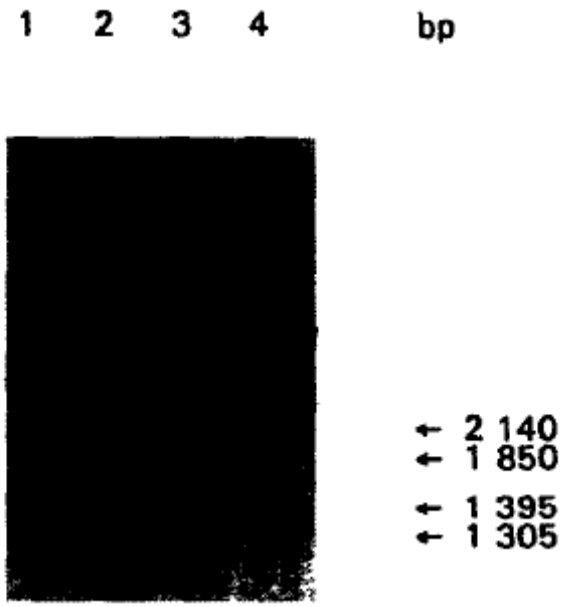

Fig. 4. Variation in ribotypes of some lactic acid bacteria used in this study. Lanes 2 - 8 Eco RI digests, Lanes 9 - 16 Hind III digest. Lanes 2 and 9 Weissella halotolerans ATCC 35410; lanes 3 and 10 W. viridescens ATCC 12706; lanes 4 and 11 Lactobacillus almentarius ATCC 29643; lanes 5 and 12 L. brevis ATCC 14869; lanes 6 and 13 L. curvatus DSM 20019; lane 14 L. farciminis DSM 20184; lanes 7 and 15 L. plantarum ATCC 14917 and lanes 8 and 16 ropy slime producing L. sake (Group 1).